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13. ABSTRACT ***(Maximum 200 Words)***
Breast cancers differ from benign breast epithelium through prominent expression of oncogenic pp32 gene family members. Whereas benign breast epithelium predominantly expresses pp32, a tumor suppressor, breast cancers express pp32r1 and pp32r2, which are oncogenic. The study purpose is to confirm and extend these preliminary results, to develop practical means to assay pp32 gene family members in clinical samples, and to determine the clinical significance of their presence in pre-invasive breast disease. The approved proposal encompassed four broad tasks: [1] characterization of the pp32 expression phenotype of a larger sample of 40 breast cancers; [2] development of a practical assay for altered pp32 transcripts in archival tissue; [3] determination of the association of specific pp32 variants in laser capture microdissected DCIS with invasion and with high-grade comedo DCIS; and [4] application of the results in a retrospective study (if [3] shows meaningful correlations) to a population of patients with follow-up. During the reporting period, we developed quantitative assays pp32, pp32r1,and pp32 mRNA copy number in breast cancer cell lines and in normal tissues demonstrating that the representation of pp32r1 and pp32r2 is relatively low in comparison to pp32. This assay is now ready for application to clinical samples.

14. SUBJECT TERMS
pathobiology; molecular pathology; translational research; tumor suppressor, pre-invasive breast disease, DCIS, prognosis

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Table of Contents

INTRODUCTION

Since this study was first proposed, parallel fundamental work in the biology of pp32 by our laboratory and others has brought pp32 to central prominence as a regulator of histone acetylation, and through this function, gene expression in a molecular sense; in a biologic sense, pp32 plays a major role in the control of cell differentiation. In turn, this knowledge provides a more compelling, if more complex rationale for the careful analysis of the pp32 gene family in cancer than existed at the outset of this study. Most importantly, it credibly points to a potential role of pp32 as a target of therapeutic intervention. To summarize a complex literature briefly, pp32 frequently co-isolates with the SET oncoprotein. The pp32-SET complex are key components of a potent inhibitor of histone acetyl transferases (1); acetylation of histones facilitates gene expression by modifying chromatin structure and making genes more accessible to transcription factors and other machinery required for gene expression. The domain of pp32 required for inhibition of histone acetylation is the same region that we identified as being required for tumor suppression (2,3). We have recently shown that antisense inhibition of pp32 drives cells to differentiate and cease proliferation; antisense inhibition of pp32 is accompanied by decreased expression of the proliferative marker PCNA, modulation of histone acetylation, expression and elaboration of autocrine differentiation factors, and acquisition of differentiation markers (4). These studies were initially carried out in human bladder carcinoma and neoplastic hematopoietic cell lines, but are now being extended to human breast cancer.

Regarding the specific project funded by USAMRMC, our previous work demonstrated that breast cancers differ from benign breast epithelium in their expression of oncogenic members of the pp32 gene family. Whereas benign breast epithelium solely expresses pp32, a tumor suppressor, breast cancers express pp32r1 and pp32r2, which are oncogenic. The approved proposal encompassed four technical objectives: [1] characterization of the pp32 expression phenotype of a larger sample of 40 breast cancers; [2] development of a practical assay for altered pp32 transcripts in archival tissue; [3] determination of the association of specific pp32 variants in laser capture microdissected DCIS with invasion and with high-grade comedo DCIS; and [4] application of the results in a retrospective study (if [3] shows meaningful correlations) to a population of patients with follow-up. Since submission and approval of the original proposal, several technical and scientific events have occurred that have positively impacted the strategy, efficiency with which the remaining aims can be accomplished, and dramatically increased the translational potential of the technology should the study establish analysis of the pp32 gene family as a clinically important assay of biologic potential in pre-invasive breast neoplasia.

BODY

Task 1. This task involves characterization of abnormal pp32 transcripts in frozen samples of human duct carcinoma compared to paired normal breast controls. Work under this approved task will entail the analysis of 40 frozen breast cancer specimens by RT-PCR, cloning, and sequencing of 10 to 20 clones of pp32 gene family members derived from the tumors to detect variant sequences. Additional features of the

task involve analysis of the frequency and position of previously uncharacterized changes.

Progress: As noted in the previous report, completion of this task had been deferred pending completion of Task 2. Further refinement of the quantitative PCR assay under Task 2 confirmed the supposition expressed last year that significant differences in expression of pp32, pp32r1, and pp32r2 would render the cloning method initially proposed inherently inaccurate.

Further efforts to develop a reliable *in situ* hybridization method as proposed last year proved unsuccessful. The likely reason lies in the close similarity of pp32 family member sequences. The usual strategy for oligonucleotide-based *in situ* hybridization is to employ multiple oligonucleotides in order to increase the signal to noise ratio. The close similarity of sequences means that there are relatively few differences to take advantage of in order to configure such an assay; indeed, only single oligonucleotides were found to be usable, and these failed to produce sufficient signal even when used in conjunction with RNA amplification. The goal proposed in the last report for completion of this task has now been modified from the previous report. When Task 2 is completed, the following strategy will be applied, which will efficiently identify candidates for subsequent sequence analysis: [1] tumor sections will be *in situ* hybridized with a 298 bp consensus probe that hybridizes with all known pp32 gene family members; [2] adjacent sections will be microdissected and quantitatively analyzed for pp32, pp32r1, and pp32r2; [3] breast carcinomas or laser capture microdissected areas of breast carcinomas will be selected for further molecular analysis if they display a phenotype of pp32 consensus + and are negative by each of the highly specific quantitative RT-PCR analyses for pp32, pp32r1, and pp32r2. This pattern would indicate the presumptive presence of novel members of the pp32 gene family. This modified approach is expected to increase the efficiency with which additional pp32 gene family members can be identified. Work on this approved Task is expected to resume shortly pending completion of the assay.

Task 2. This task involves development of a specific molecular assay compatible with use on archival tissue. The task approved in the proposal involved dissection or laser capture microdissection of tissue, RT-PCR amplification of pp32 gene family members, development of a restriction fragment length polymorphism analysis to distinguish pp32 gene family members from one another, and assay validation.

Progress: Figure 1 illustrates the completed development of the quantitative assay for pp32; analogous data exist for pp32r1 and pp32r2. Briefly, a competitor approach was developed to quantitate the expression of the three pp32-related genes. We also intended to co-reverse transcribe and then co-amplify the competitor and the endogenous sequences with a single primer set specific for each of the three pp32-related transcripts. Co-amplification of the endogenous sequence and the competitor in the same tube with one primer set eliminated the possibility of

variations in amplification efficiency between the endogenous sequence and the competitor. We first PCR synthesized three separate competitor DNA sequences with forward and reverse primer sequences specific for pp32, pp32r1 or pp32r2. The sequence internal to the primer sites was different from any pp32-related sequence although the percentage G+C and A+T was approximately the same as each of the pp32 related genes. The amplicon size from competitor was 280bp which could be easily distinguished from the amplicons generated by the endogenous genes (pp32, pp32r2 - 195bp; pp32r1 - 185bp) on 2% agarose gel electrophoresis. The competitor DNA was subcloned into an expression vector, pCR II TOPO (Invitrogen, Carlsbad, CA). Sense strand mRNA was generated from each of the three competitors utilizing T7 or Sp6 RNA polymerases and the MegaScript in vitro transcription kit (Ambion Inc., Austin, TX). The in vitro transcribed RNA was DNase treated to eliminate template DNA and absence of DNA was confirmed by PCR-only reactions without reverse transcription.

Quantitative RT-PCR was performed using the One step RT-PCR kit (Qiagen). Three tubes each with a constant amount of RNA but with varying amounts of competitor RNA were used to analyze the quantitative expression of the three pp32-related genes from each sample. The amounts of input RNA, copies of the competitor RNA, and the number of PCR cycles used are shown in the tables accompanying each graph. The RT step was carried out at 52° for 45 minutes followed by inactivation of the reverse transcription for 15 minutes at 95°. This step also simultaneously activated the HotStar Taq polymerase. Each PCR cycle consisted of denaturation at 92° for 30 sec, primer annealing at 57° for 30 sec, and an extension step at 72° for 35 sec. After a final extension step at 72° for 10 minutes, 10 µl of each cDNA product was stained with 1 µl of 1:10,000 dilution of SYBR Gold (Molecular Probes, Inc., Eugene, OR) and run on a 2% agarose gel. The size of the amplicon from the competitor RNA was 280 bp whereas the size for the amplicon from the endogenous pp32 and pp32r2 was 195bp and from pp32r1 was 185 bp. After gel electrophoresis, the gel was photographed under UV transillumination on the Strategene Eagle Eye system. The image was saved as a tiff file on a disk and analyzed for band intensities utilizing the TotalLab Software (Phoretix). Since incorporation of dye is dependent on size, the intensity of the smaller test band was multiplied by the ratio [competitor size/test amplicon size]. This was designated as the corrected test band intensity. Then, the ratio of the intensities of the competitor to the corrected test band intensity was obtained. A linear regression analysis was performed with the log copy number of the competitor on the X-axis and the log of the above ratio on the Y-axis. The point at which the regression line intersected the X-axis indicated an equal amount of starting endogenous template and exogenously added competitor.

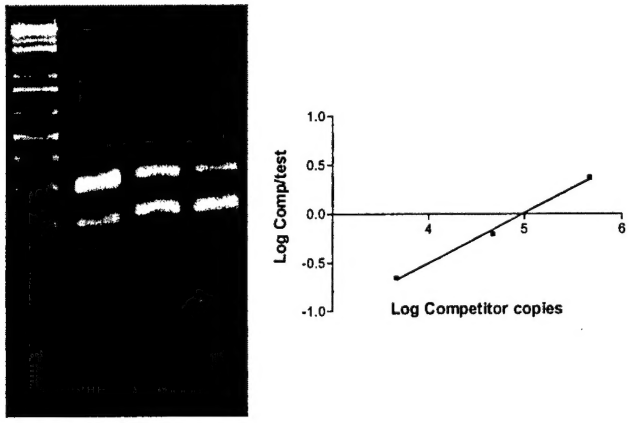

Figure 1. Representative Competitive RT-PCR Analysis of pp32. All lanes represent 20 ng of total RNA. A, 4.63 x 105 copies of competitor RNA; B, 4.63 x 104 copies; C, 4.63 x 103 copies. The graph shows the corresponding linear regression line (r2 = 0.9954, p = 0.0432).

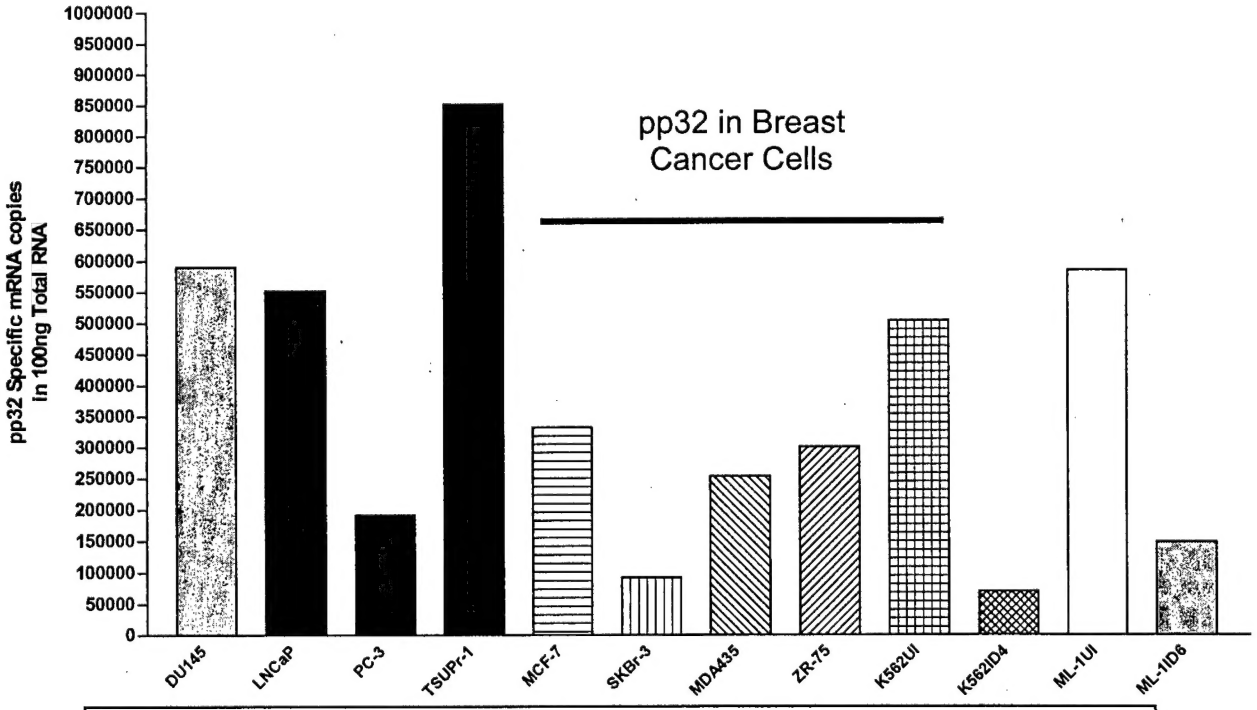

Figure 2. Quantitative Expression of pp32 in Neoplastic Cell Lines. ML1 and K562 cells both show marked reduction of pp32 expression upon differentiation.

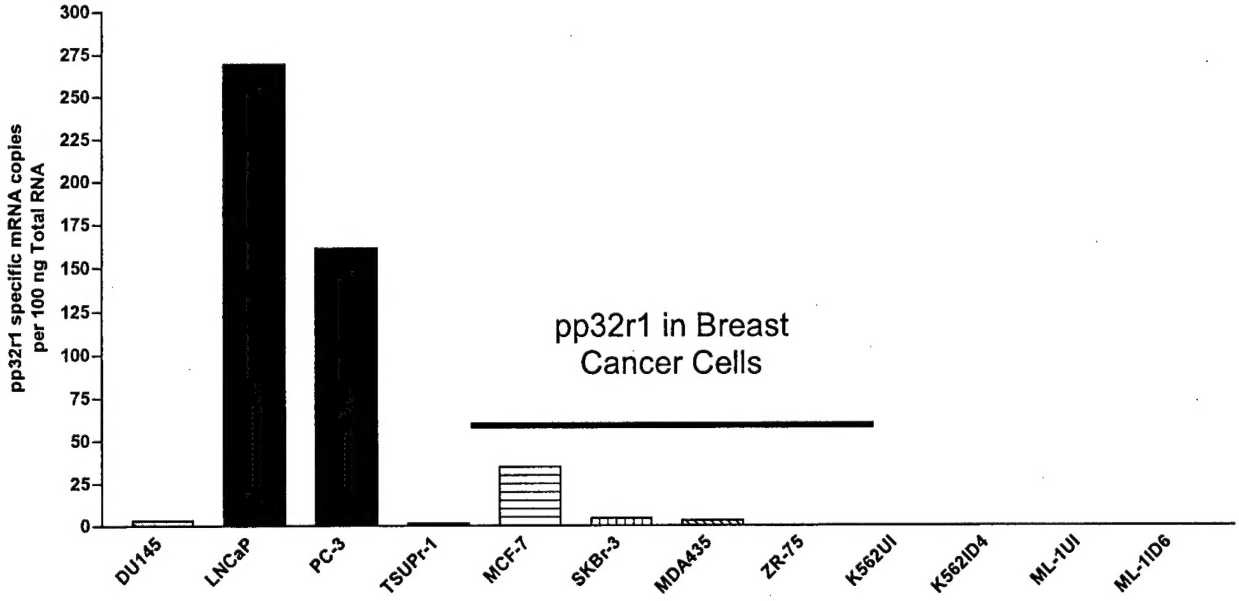

Figure 3. Quantitative Expression of pp32r1 in Neoplastic Cell Lines.

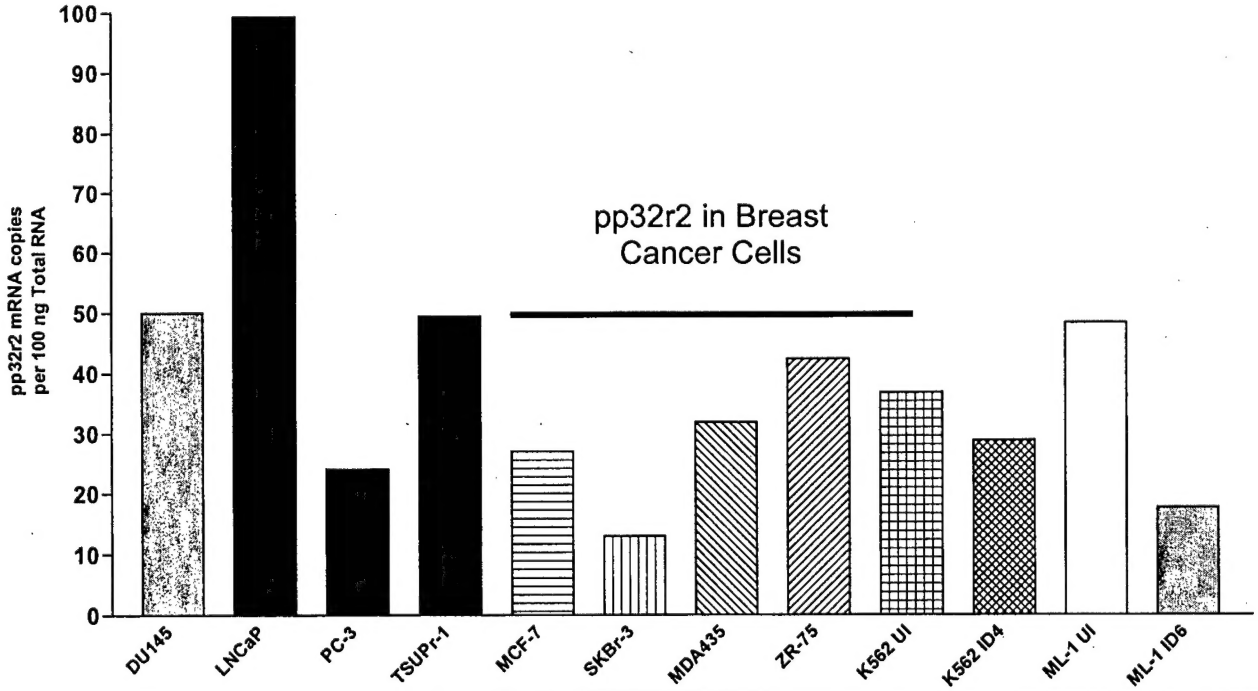

Figure 4. Quantitative Analysis of pp32r2 Expression in Neoplastic Cell Lines. Note that pp32r2 is essentially unchanged upon differentiation of K562, and only slightly reduced upon differentiation of ML-1.

The data clearly show that [1] pp32, pp32r1, and pp32r2 can be resolved from one another and quantitated, and [2] that expression differences exist among breast cancer cell lines. The short amplicons employed in this assay were specifically chosen to be compatible with the RNA that is characteristically harvested from paraffin sections.

Task 3. Identification of pp32 variants preferentially expressed in DCIS with co-existing invasive duct carcinoma or in high-grade comedo DCIS. This task involves a comparison of the expression of pp32 variants in pre-invasive breast cancer using the presence of co-existing invasive cancer as a surrogate marker for increased risk. Studies will use the method developed in Task 2 to analyze tissue provided by Dr. David Page, with subsequent statistical analysis of the results for meaningful associations.

Progress: Now that Task 2 is complete, albeit by a more circuitous route than was originally anticipated, the quantitative PCR methodology will now be applied to clinical samples as originally proposed. Work on this task is about to begin.

Task 4. Determination of the significance of variant pp32 expression as a risk factor for subsequent development of invasive breast cancer (contingent upon Task 3 results).

Progress: Work on this task will commence upon completion of Task 3.

KEY RESEARCH ACCOMPLISHMENTS

After some difficulty caused by unanticipated quantitative differences in pp32, pp32r1, and pp32r2 expression, and by technical difficulties associated with *in situ* hybridization using oligonucleotide probes, we have developed, at last, a reliable and robust quantitative assay that can now be applied to the original tasks associated with this project.

REPORTABLE OUTCOMES

The paper reported last year as in press has now been published.

Kadkol SS, Abou El Naga G, Brody JR, Bai J, Gusev Y, Dooley WC, and Pasternack, GR. Expression of pp32 gene family members in breast cancer. Breast Cancer Research and Treatment. 68:65-73, 2001

The results of the initial clinical studies will be reported at the forthcoming Era of Hope meeting.

CONCLUSIONS

New technology has been developed during the first reporting period that will greatly increase the potential impact of the studies to be carried during the remaining funding period. Work will be carried on beyond the funding period in order to complete the tasks.

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APPENDICES

N/A